Low serum levels of 25-hydroxyvitamin D predict hip fracture in the elderly. A NOREPOS study

Kristin Holvik[1,2], Luai A. Ahmed[3], Siri Forsmo[4], Clara G. Gjesdal[5,6], Guri Grimnes[7], Sven Ove Samuelsen[8,2], Berit Schei[4], Rune Blomhoff[9], Grethe S. Tell[1,2], Haakon E. Meyer[10,2]

1) Department of Public Health and Primary Health Care, University of Bergen, Norway
2) Division of Epidemiology, Norwegian Institute of Public Health, Norway
3) Department of Health and Care Sciences, Faculty of Health Sciences, University of Tromsø, Norway
4) Department of Public Health and General Practice, Norwegian University of Science and Technology, Trondheim, Norway
5) Department of Rheumatology, Haukeland University Hospital, Bergen, Norway
6) Section of Rheumatology, Institute of medicine, University of Bergen, Bergen, Norway
7) Tromsø Endocrine Research Group, Department of Clinical Medicine, University of Tromsø, Norway
8) Department of Mathematics, University of Oslo, Norway
9) Department of Nutrition, Institute of Basic Medical Sciences, University of Oslo, Norway
10) Institute of Health and Society, University of Oslo, Norway

Correspondence and requests for reprints:
Kristin Holvik, Norwegian Institute of Public Health, P.O. Box 4404 Nydalen, 0403 Oslo, Norway
E-mail address: kristin.holvik@fhi.no
Phone number: +47 - 21 07 83 97 Fax number: +47 - 22 35 36 05

Word count in abstract: 244
Word count in main text (excl. abstract, tables, figures, references): 3519
Number of tables: 3 Number of figures: 3
Number of references: 40
Key terms: Hip fracture, vitamin D status, 25-hydroxyvitamin D, case-cohort, Norway

Ethical approval

The four regional parts of this study were approved by the respective Regional Committees for Medical and Health Research Ethics (REC): REC West, ref. 067.09; REC Central, ref. 2009-714/2; REC North, ref. 31/94; REC South East, ref. 08/2037-4.

Authors' contribution

All study centers providing data are represented among the co-authors. KH, SF, CGG, GG, BS, GST and HEM contributed to developing the protocol and obtain funding. KH prepared the data, performed the statistical analyses and drafted the manuscript. SOS provided statistical advice. All co-authors have critically discussed the results, revised the manuscript and accepted the final version.

Disclosure statement

KH, LAA, SF, CGG, GG, SOS, BS and GST have nothing to declare. RB has interests in Bioindex AS and Vitas AS. Bioindex was established by Birkeland Innovation, the Technology transfer office at the University of Oslo while Vitas was established by Oslo Innovation Center. HEM contributes to a randomized trial of vitamin D supplementation sponsored by the University of Oslo and The Norwegian Women's Public Health Association. The trial is also supported by Fürst Medical Laboratory and Nycomed.

Acknowledgements

This study was funded by a grant from the Research Council of Norway. The serum sample analyses in HUNT 2 were partly funded by a grant from Central Norway Regional Health Authority. We would like to acknowledge the people involved in carrying out the data collection in Tromsø IV, HUNT 2, HUSK, and HUBRO, those involved in establishing and maintaining the four respective hip fracture follow-up registers, those involved in data management, those involved in biobanks and blood sample handling, and the laboratory AS Vitas, Oslo, Norway, for performing the serum sample analyses. Finally, we would like to thank the participants in the health studies in Norway.

ABSTRACT

Background: Despite considerable interest, the relationship between circulating 25-hydroxyvitamin D and risk of hip fracture is not fully established.

Objective: To study the association between serum 25-hydroxyvitamin D concentrations (s-25(OH)D) and risk of hip fracture in Norway, a high-latitude country that has among the highest hip fracture rates worldwide.

Methods: N=21,774 men and women aged 65-79 attended four community-based health studies during 1994-2001. Information on subsequent hip fractures were retrieved from electronic hospital discharge registers, with maximum follow-up 10.7 years. Using a stratified case-cohort design, s-25(OH)D was determined by HPLC-APCI-MS in stored serum samples in hip fracture cases (n=1175; 307 men, 868 women) and in gender-stratified random samples (n=1438). Cox proportional hazards regression adapted for the case-cohort design was performed.

Results: We observed an inverse association between s-25(OH)D and hip fracture; those with s-25(OH)D in the lowest quartile (<42.2 nmol/l) had a 38% (95% CI 9-74%) increased risk of hip fracture compared with the highest quartile (>=67.9 nmol/l) in a model accounting for age, gender, study center, and BMI. The association was stronger in men than in women: HR 1.65 (95% CI 1.04-2.61) versus HR 1.25 (95% CI 0.95-1.65).

Conclusion: In this prospective case-cohort study of hip fractures, the largest ever reported, we found an increased risk of hip fracture in subjects in the lowest compared to the highest quartile of serum 25-hydroxyvitamin D. In accordance with findings of previous community-based studies, low vitamin D status was a modest risk factor for hip fracture.

INTRODUCTION

The highest incidence rate of hip fractures worldwide has been reported in Oslo, Norway (1, 2). The causes of these high rates in Norway, and in the other Scandinavian countries, are not known (1). Considerable attention has been given to the relation between low vitamin D status and osteoporotic fractures.

Vitamin D is essential for intestinal calcium absorption and for maintaining calcium homeostasis and skeletal integrity (3). A well-known consequence of vitamin D deficiency is secondary hyperparathyroidism which may lead to bone resorption, osteoporosis, and increased fracture risk (3). Some (4-6) but not other (7, 8) studies have found a positive association between vitamin D status and bone mineral density (BMD) in Caucasian populations. It has therefore been suggested that vitamin D may not be a major determinant of bone health in populations with generally adequate vitamin D and calcium status (7, 8). However, vitamin D may not influence fracture risk through bone metabolism only, but also through muscle function and risk of falling (9).

Most prospective studies investigating the role of vitamin D status in fracture risk included a low number of hip fractures and analyzed all fractures as outcome (7, 10-14). Some have not been able to establish any association (7, 12) . However, an increased fracture risk has been reported at 25(OH)D levels below 30 nmol/l in the Netherlands (13), and below 40 nmol/l or 50 nmol/l in Sweden (11, 14). The association between s-25(OH)D and hip fracture (as the main outcome) has been studied in three prospective studies from the US: The NHANES III (15), the MrOS study in men (16), and the Women's Health Initiative (17), the latter being the largest prospective study we are aware of with vitamin D status in blood determined at baseline and hip fracture as outcome, including 400 cases. They observed a statistically significant trend of increasing risk of hip fracture through decreasing quartiles of 25(OH)D.

Meta-analyses of randomized controlled trials conclude that supplementation with vitamin D in combination with calcium has a modest preventive effect on hip fracture, whereas a preventive effect

has not been established for vitamin D alone (18-20). In a re-analysis of data from 11 RCTs, only the highest actual intake levels (>=792 IU/day) were significantly associated with reduced risk of hip fracture (21).

The aim of this study was to investigate the association between serum 25-hydroxyvitamin D levels (the sum of $25(OH)D_2 + 25(OH)D_3$) and risk of hip fracture during up to 11 years follow-up in community-dwelling older adults in the country with the world's highest hip fracture incidence.

MATERIALS AND METHODS

As part of the Norwegian Epidemiologic Osteoporosis Studies (NOREPOS) research collaboration (www.norepos.no), we performed a gender-stratified case-cohort study (22, 23) using baseline data from four population-based studies in Norway: The Tromsø Study (Tromsø IV) (latitude 69°N) in 1994-95 (www.tromsostudy.com), The Nord-Trøndelag Health Study (HUNT 2) (63-65°N) in 1995-97 (www.ntnu.edu/hunt), The Hordaland Health Study (HUSK) (60°N) in 1997-99 (husk-en.b.uib.no), and The Oslo Health Study (HUBRO) (59°N) in 2000-01 (www.fhi.no/hubro-en).

Study population

The total cohort included 21,774 home-dwelling men and women aged 65-79 years. The lower age limit was set to define an elderly group, and the higher age limit was set to reduce censoring due to deaths. In Tromsø IV and HUNT 2, age distribution of participants at baseline was 65-79 years, while the participants in HUSK comprised individuals age 70-73, and in HUBRO age 75-77.

Baseline information

Baseline examinations included collection of non-fasting blood samples which were frozen and stored, measurements of weight, height and blood pressure, and self-administered questionnaires. The four studies are part of Cohort of Norway (CONOR) (24), thus standardized questionnaire and background data were available. Questionnaires included information on previous fracture, physical activity, cigarette smoking, chronic diseases, self-perceived health, and use of medications. Body mass index (BMI, kg/m^2) was calculated. A question on current health status with four response alternatives was dichotomized into a variable indicating good/very good vs. poor/not so good self-rated health. Participants answering "yes" to the question "Do you currently smoke cigarettes daily?" were defined as smokers. Participants were categorized as physically active or inactive according to their response on two four-graded questions concerning weekly frequency of hard and moderate physical activity during leisure time. Education was assessed as number of years completed education. Questions on whether the participant suffered or had ever suffered from chronic diseases, including osteoporosis, or had experienced adverse events, including fracture of the wrist or hip, were answered with yes or no.

Open-ended questions about medication use were included in HUBRO, HUSK, and Tromsø IV, and were coded according to the Anatomical Therapeutic Chemical Classification (ATC) System. Those reporting medications with ATC codes G03 C and F were defined as estrogen therapy users. In HUNT 2, only women below 70 years were inquired about estrogen therapy, and those responding “currently” were defined as current users. In addition, HUBRO included the question “Do you or have you used oestrogen (tablets or patches)”, and all those answering "yes, currently" to this question were also included in the estrogen therapy users category. While 99.0% (1162 cases and 1338 non-cases) had valid measurements of weight and height, the response rates on questionnaire data ranged from 98.5% (self-rated health) to 77.6% (physical activity).

Deaths and emigration

Dates of death or emigration were obtained from the Norwegian Population Register and follow-up time was calculated. End of follow-up was 31.12.2004 in Tromsø IV and HUNT 2, 31.12.2007 in HUBRO, and 31.12.2008 in HUSK, yielding a maximum follow-up of 10.7 years.

Identification of hip fractures

Information about hip fractures was obtained from fracture registers at each study site. A hip fracture was defined as the first fracture of the proximal femur occurring during the observation period. In Tromsø IV and HUNT 2, cases included the diagnoses of fracture of the femoral neck and pertrochanteric fracture, while the case definition in HUSK and HUBRO also included subtrochanteric hip fractures. The discharge diagnoses used to classify a hip fracture were according to the International Classification of Diseases, Ninth Revision (ICD-9): 820-820.9 and Tenth Revision (ICD-10): S72.0, S72.1 and S72.2.

In Tromsø IV, hip fractures subsequent to the baseline examination until moving out of Tromsø municipality or end of follow-up were retrieved from the radiographic archives and validated against the patient discharge records at the University Hospital of Northern Norway, Tromsø (25). In HUNT 2, hip fractures treated in Levanger or Namsos hospitals, the two only hospitals in Nord-Trøndelag,

were obtained from the electronic registers of the Nord-Trøndelag Hospital Trust (26). In HUSK, the computerized discharge diagnoses records of the six hospitals serving Hordaland County were searched for diagnosis codes. All admittances with diagnosis codes indicating femoral fracture with a corresponding surgical procedure indicating a primary operation for hip fracture were defined as incident hip fractures (27). In HUBRO, hip fractures were retrieved by linkage to the electronic discharge registers in the five hospitals treating hip fractures in Oslo, by performing a broad search on all diagnosis codes for femur fractures as well as surgical procedure codes for fracture surgery in femur and hip arthroplasty. True primary hip fractures were confirmed by reviewing patients' medical records, except in one hospital where only 42% of the retrieved hospital stays were verified in medical records, as the remainder were no longer electronic accessible. For 84 (16.2%) admissions not reviewed in medical records, a hip fracture diagnosis was confirmed if the hospital stay included a surgical procedure characteristic for a primary operation for hip fracture, corresponding to the method used in HUSK.

Selection of cases and the subcohort

All participants who suffered a hip fracture during follow-up were included as cases. Participants with self-reported previous fractures or fractures occurring prior to their date of attendance, but not during the observation period, were treated as non-cases. The gender-specific subcohorts were defined as random unmatched samples of 4.5% of men and 9.0% of women in the study population at baseline. These inclusion fractions were chosen so that the subcohorts within each gender would be approximately the same size as the number of cases.

Serum sample analyses

Frozen serum samples were sent to AS Vitas, Oslo, Norway, where they were analyzed in 2011. Samples from cases and non-cases at each study center were analyzed simultaneously. Biobank staff and laboratory staff were blinded with regard to case status. 25-hydroxyvitamin D_2 and 25-hydroxyvitamin D_3 in serum were determined by HPLC-APCI- MS (28). One hundred and fifty µL of human plasma was diluted with 450 µL 2-propanol containing BHT as an antioxidant. After thorough

mixing (15 min) and centrifugation (10 min, 4000 g at 10 °C), an aliquot of 35 µL was injected from the supernatant into the HPLC system. HPLC was performed with a HP 1100 liquid chromatograph (Agilent Technologies, Palo Alta, CA, USA) interfaced by atmospheric pressure chemical ionization (APCI) to a HP mass spectrometric detector (MS) operated in single ion monitoring mode (SIM). Vitamin D analogues were separated on a 4.6 mm x 50 mm reversed phase column with 1.8 µM particles. The column temperature was 80°C. A two-point calibration curve was made from analysis of albumin solution enriched with known vitamin D concentration. Recovery is 95%, the method is linear from 5-400 nM at least and the limit of detection is 1-4 nM. CVs for interassay analyses were 7.6% at s-25(OH)D 47.8 nmol/l and 6.9% at s-25(OH)D 83.0 nmol/l.

Statistical analyses

Data management was done in PASW Statistics 17 / IBM SPSS 20, and main statistical analyses were performed in R (29). We performed Cox proportional hazards regression adjusted for a gender-stratified case-cohort design (22, 23), using the case-cohort function 'cch' in the R package 'survival' (30). Age is accounted for through the flexible baseline in Cox regression. We initially performed Cox regression with penalized splines of s-25(OH)D (31) to examine the trends in hip fracture hazard over the full distribution of s-25(OH)D. In addition, we examined hazard ratios according to quartiles of s-25(OH)D. Quartile limits were based on distribution of 25(OH)D in the subcohort. Gender-specific quartile limits were used in the gender-specific analyses. Additional analyses were performed using pooled month-specific quartiles of s-25(OH)D to account for seasonal variation (32). We also performed a subgroup analysis including only those who did not report prevalent osteoporosis at baseline examination, and we performed additional study center-specific analyses where we scrutinized the association between s-25(OH)D and hip fracture within each study center. Potential confounders considered were BMI, cigarette smoking, physical inactivity, education, and self-rated health. Tests and plots of Schoenfeld residuals (31) revealed non-proportional hazards for study center, suggesting a time-dependent effect of study center on hip fracture hazard. Using strata allowing for different baseline hazards by study center yielded virtually identical results as when including the variable as a factor. Study center is therefore included as an adjustment variable.

RESULTS

Incident hip fractures

During a median observation time of 8.2 years, 1232 individuals (340 men (3.4%) and 892 women (7.5%)) suffered a hip fracture. The randomly sampled subcohorts included 1502 individuals of whom 93 became cases. Intact frozen serum samples were obtained and successfully analyzed for 2526 (95.6% of those selected) participants. Missing serum was equally distributed among cases and non-cases. The number included in the presented analyses are 2500 with valid BMI data (94.7% of those selected), of whom 1162 hip fracture cases (Fig. 1).

Characteristics of the study population

Baseline characteristics according to whether participants suffered a hip fracture or not during the observation period are shown in Table 1. Cases were on average older at baseline, and a higher proportion of cases reported previous fractures. Among women, cases also had significantly lower BMI, poorer self-rated health, higher smoking prevalence, and a higher prevalence of osteoporosis.

Serum 25-hydroxyvitamin D

Overall median (25,75-percentile) s-25(OH)D in the randomly sampled subcohort was 53.5 (42.2, 67.8) nmol/l. S-25(OH)D was slightly higher in men than in women (median 55.5 vs. 52.5 nmol/l, p=0.07). It was similar across study centers except in HUBRO where it was higher (median 61.6 nmol/l, $p<0.001$ for HUBRO vs. the other study centers). Baseline characteristics in the subcohorts according to quartiles of s-25(OH)D are shown in Table 2. In men, participants were older in the highest s-25(OH)D quartile, and the prevalence of smoking and physical inactivity was lower at higher s-25(OH)D although this was not statistically significant. In women there was a clear inverse relationship between BMI and s-25(OH)D (Spearman's rho= −0.11 (p=0.001)).

Risk of hip fracture according to serum 25-hydroxyvitamin D

We observed an inverse association between s-25(OH)D and hazard of hip fracture (Fig. 2), with increasing risk estimates across decreasing quartiles of s-25(OH)D (Table 3). Cox regression with

penalized splines revealed a linear association between s-25(OH)D and HR of hip fracture (p=0.008). When entering s-25(OH)D as a continuous variable, HR (95% CI) was 1.13 (1.03, 1.25) per 25 nmol/l lower s-25(OH)D in a model accounting for age, gender, study center, and BMI (p=0.014).

Those with s-25(OH)D in the lowest quartile (<42.2 nmol/l) had a statistically significant 38% (95% CI 9-74%) increased risk of hip fracture compared with the highest quartile (>=67.9 nmol/l) in a model accounting for age, gender, study center, and BMI (Table 3). The trend across quartiles was statistically significant (p=0.009). The association was statistically significant in men (HR 1.65, 95% CI 1.04-2.61) but not in women (HR 1.25, 95% CI 0.95-1.65).

The interaction term between gender and s-25(OH)D on HR of hip fracture was not statistically significant. The association in the genders combined analysis persisted after adjustment for calendar month of blood sample (HR 1.34, 95% CI 1.05-1.70). Further adjustment for cigarette smoking, physical inactivity and self-rated health yielded similar results. In additional analysis using month-specific quartiles of s-25(OH)D as exposure (32), HR was 1.32 (95% CI 1.04-1.67) in men and women combined in a model including age, gender, study center, and BMI.

Excluding those with self-reported osteoporosis at baseline strengthened the association somewhat: HR 1.49 (95% CI 1.16-1.91) for Q1 vs. Q4 in genders combined, and HR 1.34 (95% CI 1.00-1.81) in women, in the model including age, study center, and BMI. The association in men was not influenced by excluding those with osteoporosis due to the very low prevalence of osteoporosis in men.

In additional analyses restricted to each study center, a similar association between s-25(OH)D and risk of hip fracture was observed within all study centers. The risk estimates were higher in men than in women, except in Tromsø where the risk estimate was higher in women. However, the only significant associations were seen in HUBRO (180 hip fractures) where the overall HR (95% CI) for Q1 vs. Q4 was 2.03 (1.12-3.69) in a BMI-adjusted model.

DISCUSSION

In this large case-cohort study we observed a modest inverse association between s-25(OH)D and hip fracture during up to 10.7 years of follow-up. In our community-dwelling elderly population, there was a 38% (95% CI 9-74%) increased risk of hip fracture in the lowest (<42.2 nmol/l) compared with the highest quartile (>=69.7 nmol/l) of 25-hydroxyvitamin D after accounting for the effects of age, gender, study center, and BMI.

In order to appraise our results relative to previous studies, we have plotted relative risks with 95% confidence intervals for hip fracture at 25-hydroxyvitamin D concentrations below 50 nmol/l in prospective studies including our own (Fig. 3). In addition to our study, we identified published data from five prospective studies on baseline s-25(OH)D in a total of 843 Caucasian subjects with hip fracture (216 men and 627 women) (14-17, 33). Variations in biochemical assays, statistical adjustments, age differences and other discrepancies between populations may hamper comparisons between studies. However, all published studies reporting relative risk estimates for hip fractures found estimates in favour of an increased risk at lower 25-hydroxyvitamin D, although with varying precision.

There is a large variation in 25-hydroxyvitamin D concentrations from different assays and laboratories in the same blood samples (34, 35). We therefore chose to present hazard ratios according to relative distribution (quartiles) rather than absolute limits. In our data, the 25- and 75-percentile (42.2 and 67.8 nmol/l) fell close to the commonly used cutoffs for defining vitamin D insufficiency or sufficiency; 50 and 75 nmol/l. As expected, in additional analyses with these commonly accepted limits, the results were similar, with a statistically significant 35% increased risk in subjects with levels <50 compared with >75 nmol/l when adjusting for gender, study center and BMI. These results suggest a preventive effect of levels above 75 nmol/l compared with levels below 50 nmol/l. Hip fracture risk decreased continuously with increasing s-25(OH)D, with the steepest and most consistent decrease at levels in the magnitude of 40-60 nmol/l (Fig. 2). Additional analyses yielded significantly

increased hazard ratios for hip fracture at levels <50 compared with 50-75 nmol/l (HR 1.21, 95% CI 1.01-1.45), but not at levels 50-75 nmol/l compared with >75 nmol/l (HR 1.12, 95% CI 0.88-1.42).

Excluding those reporting osteoporosis at baseline strengthened the association between low 25-hydroxyvitamin D and risk of hip fracture somewhat. Current guidelines for treatment of osteoporosis recommend daily vitamin D supplementation in combination with calcium, due to its well-documented fracture prevention effect (18, 19). Women with diagnosed osteoporosis may have improved their vitamin D status. The prevalence of osteoporosis was as high in the highest as in the lowest quartile of s-25(OH)D (Table 2B). The finding of a U-shaped association between 25(OH)D status and frailty in older women participating in the Study of Osteoporotic Fractures (36) also supports this notion.

Osteoporosis is a multifactorial disorder, and vitamin D may not be a major determinant of bone strength in populations with generally adequate vitamin D and calcium status. In spite of living at high latitude, the population in Norway has earlier been shown to have a relatively good vitamin D status (37), likely owing to the country's coastal location with strong traditions of fatty fish and cod liver oil consumption, as well as an active sun-seeking attitude. Nutritional factors other than vitamin D may also affect fracture risk and may differ between populations. Fatty fish and cod liver oil are rich in retinol, a substance that may reduce the effect of the active vitamin D hormone through competition for the nuclear receptor. As retinol intake is high in Scandinavian countries, it would be of interest to study whether high retinol influences the observed association between serum 25(OH)D and hip fracture.

Strengths and limitations

The strengths of this study include the population-based approach, inviting age-specific samples of the general population, and the prospective design with up to 10.7 years follow-up. The representativeness is, however, limited by the participation rates. These varied between the study centers. In the age groups included in this study, the overall attendance rates were 82% in Tromsø IV, 81% in HUNT 2, 77% in HUSK, and 53% in HUBRO.

As is common in cohort studies, the categorization of exposure was based on a single baseline measurement. However, data from the Tromsø study suggest a satisfactory tracking of individual 25(OH)D levels in serum over 14 years, similar to that of blood pressure and serum lipids (38).

The large number of events is a major strength. As far as we are aware, this is the largest existing study with hip fracture outcome and biochemical measurements at baseline, with serum sample analyses and a large number of covariates available for 1175 subjects (868 women and 307 men) who suffered a hip fracture during the observation period. We thus had sufficient statistical power to detect a risk increase of 30% in the lowest quartile of 25-hydroxyvitamin D compared with the three higher quartiles.

Another strength is that the measures of exposure and outcome were objective and retrieved from independent sources outside the baseline data collection settings. All serum samples were analyzed in the same laboratory, by HPLC-APCI-MS, a highly valid 25(OH)D assay (34, 39). Samples from cases and non-cases were handled and analyzed simultaneously, biobank and laboratory personnel being blinded to case status.

Heterogeneity of different health studies included in a multicenter study may introduce limitations. In this study, HR of hip fracture did not differ significantly according to study center (not shown), and analyses stratified by study center revealed the same trend in the association between s-25(OH)D and hip fracture in all regions.

The four health studies are part of the CONOR collaboration (24) and were therefore coordinated to a large extent. Some variables were collected in a standard way, such as blood samples, anthropometric measurements, and common questions including physical activity, education, chronic diseases, and self-rated health. A limitation is that we had poor data on diet and supplement use, and were unable to assess the influence of dietary calcium intake. However, results from previous studies (14, 15, 17)

suggest that taking into account variations in dietary calcium intake in a population of home-dwelling elderly men and women in an observational cohort study would not influence the observed associations substantially.

Conclusion

In this prospective case-cohort study, the largest of its kind, we found an increased risk of hip fracture in subjects in the lowest quartile of serum 25-hydroxyvitamin D compared with the highest quartile. In accordance with findings of previous community-based studies, low vitamin D status was a modest risk factor for hip fracture in our population.

TABLE 1. Baseline characteristics comparing participants who experienced a hip fracture with participants who were free of hip fracture until end of follow-up. A NOREPOS study

	Men				Women			
	n	No hip fracture[1] n=400	Hip fracture n=307	p value[2]	n	No hip fracture[1] n=951	Hip fracture n=868	p value[2]
Age, mean (SD) years	707	71.8 (3.9)	73.1 (3.2)	<0.001	1819	72.2 (3.9)	73.5 (3.4)	<0.001
s-25(OH)D, mean (SD) nmol/l	707	57.8 (20.5)	55.9 (19.8)	0.20	1819	55.7 (21.1)	55.2 (21.7)	0.64
Body mass index, mean (SD) kg/m2	701	26.4 (3.8)	25.9 (3.8)	0.12	1799	27.4 (4.5)	25.8 (4.4)	<0.001
Body height, mean (SD) cm	703	173.7 (5.8)	174.5 (6.4)	0.065	1801	159.9 (5.8)	160.2 (6.1)	0.45
Education, mean (SD) years	639	8.9 (4.1)	8.3 (3.5)	0.070	1526	7.5 (3.1)	7.5 (3.1)	1.00
Good or very good self-rated health, n (%)	700	235 (59.2)	163 (53.8)	0.18	1788	506 (54.1)	393 (46.1)	0.001
Daily cigarette smoker, n (%)	681	88 (22.8)	82 (27.8)	0.16	1745	165 (18.3)	202 (24.0)	0.004
Inactive during leisure time, n (%)	591	20 (6.0)	21 (8.2)	0.36	1368	96 (13.1)	98 (15.4)	0.27
History of hip fracture, n (%)	616	10 (2.8)	18 (6.9)	0.026	1455	39 (5.1)	71 (10.4)	<0.001
History of wrist fracture, n (%)	616	32 (8.9)	40 (15.6)	0.016	1511	220 (27.6)	285 (39.9)	<0.001
Osteoporosis, n (%)	597	5 (1.5)	3 (1.2)	1.00	1453	84 (11.1)	164 (23.6)	<0.001
Estrogen therapy, n (%)	-	-	-	-	1819	79 (8.3)	71 (8.2)	0.99

[1] Subcohort excluding cases (16 men, 71 women)

[2] ANOVA for continuous variables, chi square test for factor variables. Fisher's Exact test for osteoporosis in men due to low cell counts.

TABLE 2A. Baseline characteristics of male subcohort across quartiles of serum 25-hydroxyvitamin D. A NOREPOS study

	n	*1st Quartile 9.9-43.3 nmol/l n=104*	*2nd Quartile 43.6-55.4 nmol/l n=104*	*3rd Quartile 55.6-69.1 nmol/l n=104*	*4th Quartile 69.2-156.4 nmol/l n=104*	*p value*[1]
S-25(OH)D, mean (SD) nmol/l	416	34.0 (7.5)	50.0 (7.5)	61.7 (4.1)	83.7 (15.9)	<0.001
Age, mean (SD) years	416	71.9 (4.0)	72.1 (3.8)	71.0 (3.6)	72.5 (4.0)	0.044
Study center:						
Tromsø (Tromsø IV), n (%)	416	11 (10.6)	15 (14.4)	13 (12.5)	8 (7.7)	
Nord-Trøndelag (HUNT 2), n (%)		68 (65.4)	61 (58.7)	65 (62.5)	58 (55.8)	
Hordaland (HUSK), n (%)		19 (18.3)	14 (13.5)	16 (15.4)	14 (13.5)	
Oslo (HUBRO), n (%)		6 (5.8)	14 (13.5)	10 (9.6)	24 (23.1)	0.041
Season of blood sample:						
Summer; Apr-Sep, n (%)	416	35 (33.7)	44 (42.3)	51 (49.0)	48 (46.2)	
Winter; Oct-Mar, n (%)		69 (66.3)	60 (57.7)	53 (51.0)	56 (53.8)	0.13
Body mass index, mean (SD) kg/m2	412	27.0 (4.3)	26.1 (3.4)	25.9 (4.0)	26.3 (3.4)	0.22
Body height, mean (SD) cm	414	173.0 (5.9)	173.8 (5.6)	174.0 (5.6)	174.0 (6.1)	0.56
Education, mean (SD) years	381	8.6 (4.0)	9.1 (4.1)	9.1 (4.3)	8.6 (3.9)	0.69
Self-rated health, good or very good, n (%)	413	55 (53.4)	62 (59.6)	67 (65.0)	58 (56.3)	0.36
Daily cigarette smoker, n (%)	401	26 (25.5)	26 (26.8)	25 (25.5)	15 (14.4)	0.12
Inactive during leisure time, n (%)	349	8 (9.9)	6 (6.9)	3 (3.2)	3 (3.4)	0.21
History of hip fracture, n (%)	372	0	4 (4.3)	3 (3.3)	3 (3.2)	0.22
History of wrist fracture, n (%)	374	7 (7.6)	11 (11.8)	6 (6.3)	12 (12.8)	0.36
Osteoporosis, n (%)	357	1 (1.1)	1 (1.2)	1 (1.1)	2 (2.2)	0.94

[1] ANOVA for continuous variables, Chi Square test for factor variables. Fisher's Exact test for physical inactivity, history of hip fracture, and osteoporosis in men due to low cell counts.

TABLE 2B. Baseline characteristics of female subcohort across quartiles of serum 25-hydroxyvitamin D. A NOREPOS study

	n	*1st Quartile 4.5-41.4 nmol/l n=256*	*2nd Quartile 41.6-52.5 nmol/l n=255*	*3rd Quartile 52.5-67.0 nmol/l n=255*	*4th Quartile 67.0-193.9 nmol/l n=256*	*p value*[1]
S-25(OH)D, mean (SD) nmol/l	1022	32.2 (6.4)	47.1 (3.0)	59.5 (4.2)	84.2 (15.1)	
Age, mean (SD) years	1022	72.5 (3.9)	71.8 (4.0)	72.4 (3.7)	72.6 (3.7)	0.10
Study center:						
Tromsø (Tromsø IV), n (%)	1022	24 (9.4)	41 (16.1)	30 (11.8)	21 (8.2)	
Nord-Trøndelag (HUNT 2), n (%)		161 (62.9)	149 (58.4)	149 (58.4)	132 (51.6)	
Hordaland (HUSK), n (%)		45 (17.6)	31 (12.2)	37 (14.5)	45 (17.6)	
Oslo (HUBRO), n (%)		26 (10.2)	34 (13.3)	39 (15.3)	58 (22.7)	<0.001
Season of blood sample:						
Summer; Apr-Sep, n (%)	1022	92 (35.9)	105 (41.2)	107 (42.0)	128 (50.0)	
Winter; Oct-Mar, n (%)		164 (64.1)	150 (58.8)	148 (58.0)	128 (50.0)	0.014
Body mass index, mean (SD) kg/m2	1010	27.9 (4.8)	27.6 (4.3)	27.0 (4.6)	26.7 (4.3)	<0.001
Body height, mean (SD) cm	1011	159.8 (6.0)	160.2 (5.8)	160.1 (6.0)	159.9 (5.5)	0.80
Education, mean (SD) years	861	7.3 (2.8)	7.4 (3.0)	7.6 (3.2)	7.7 (3.2)	0.32
Self-rated health, good or very good, n (%)	1005	120 (47.8)	137 (54.2)	128 (51.4)	147 (58.3)	0.11
Daily cigarette smoker, n (%)	972	47 (19.3)	44 (18.1)	42 (17.4)	49 (20.0)	0.88
Inactive during leisure time, n (%)	779	32 (17.4)	29 (15.5)	22 (11.2)	23 (10.9)	0.16
History of hip fracture, n (%)	829	14 (7.0)	7 (3.3)	15 (7.5)	12 (5.6)	0.25
History of wrist fracture, n (%)	857	53 (25.6)	59 (26.8)	64 (30.6)	65 (29.4)	0.65
Osteoporosis, n (%)	817	26 (13.1)	27 (12.7)	17 (8.8)	28 (13.2)	0.47
Estrogen therapy, n (%)	1022	19 (7.4)	15 (5.9)	22 (8.6)	25 (9.8)	0.41

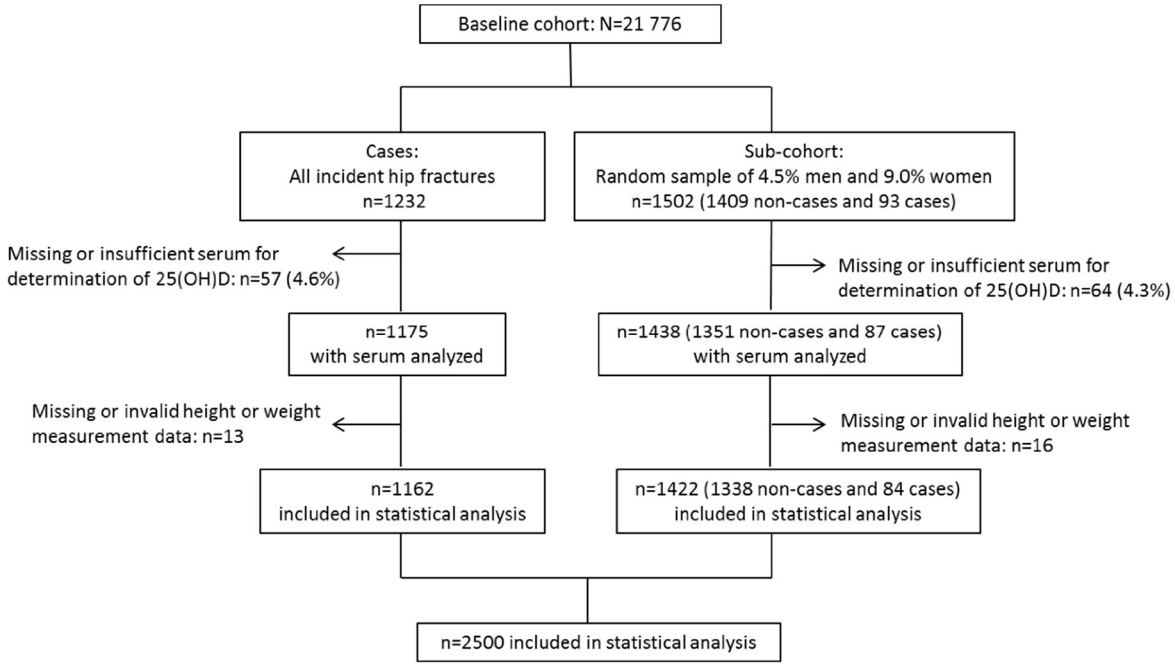
[1] ANOVA for continuous variables, chi square test for factor variables.

TABLE 3. Hazard ratios for hip fracture according to quartiles of serum 25-hydroxyvitamin D concentration (nmol/l) in NOREPOS. Cox proportional hazards regression adjusted for a gender-stratified case-cohort design. A NOREPOS study [a)]

	25(OH)D range, nmol/l	# hip fx	HR[1]	95% CI[1]	HR[2]	95% CI[2]	HR[3]	95% CI[3]
All								
Q1	4.5-42.1	317	1.27	1.01-1.59	1.38	1.09-1.74	1.34	1.05-1.70
Q2	42.2-53.5	294	1.13	0.90-1.43	1.16	0.92-1.46	1.13	0.90-1.44
Q3	53.5-67.8	272	1.13	0.90-1.42	1.12	0.88-1.41	1.10	0.87-1.39
Q4	67.9-250.0	279	1.00	(ref.)	1.00	(ref.)	1.00	(ref.)
Men								
Q1	9.9-43.3	82	1.56	0.99-2.46	1.65	1.04-2.61	1.55	0.95-2.51
Q2	43.6-55.4	74	1.29	0.82-2.01	1.27	0.81-1.99	1.24	0.77-1.98
Q3	55.6-69.1	69	1.10	0.69-1.74	1.06	0.66-1.69	1.07	0.66-1.73
Q4	69.2-156.4	79	1.00	(ref.)	1.00	(ref.)	1.00	(ref.)
Women								
Q1	4.5-41.5	227	1.14	0.87-1.48	1.25	0.95-1.65	1.23	0.92-1.63
Q2	41.6-52.5	208	1.06	0.81-1.38	1.10	0.84-1.45	1.09	0.82-1.44
Q3	52.5-67.0	212	1.12	0.86-1.47	1.16	0.88-1.52	1.14	0.86-1.49
Q4	67.0-250.0	211	1.00	(ref.)	1.00	(ref.)	1.00	(ref.)

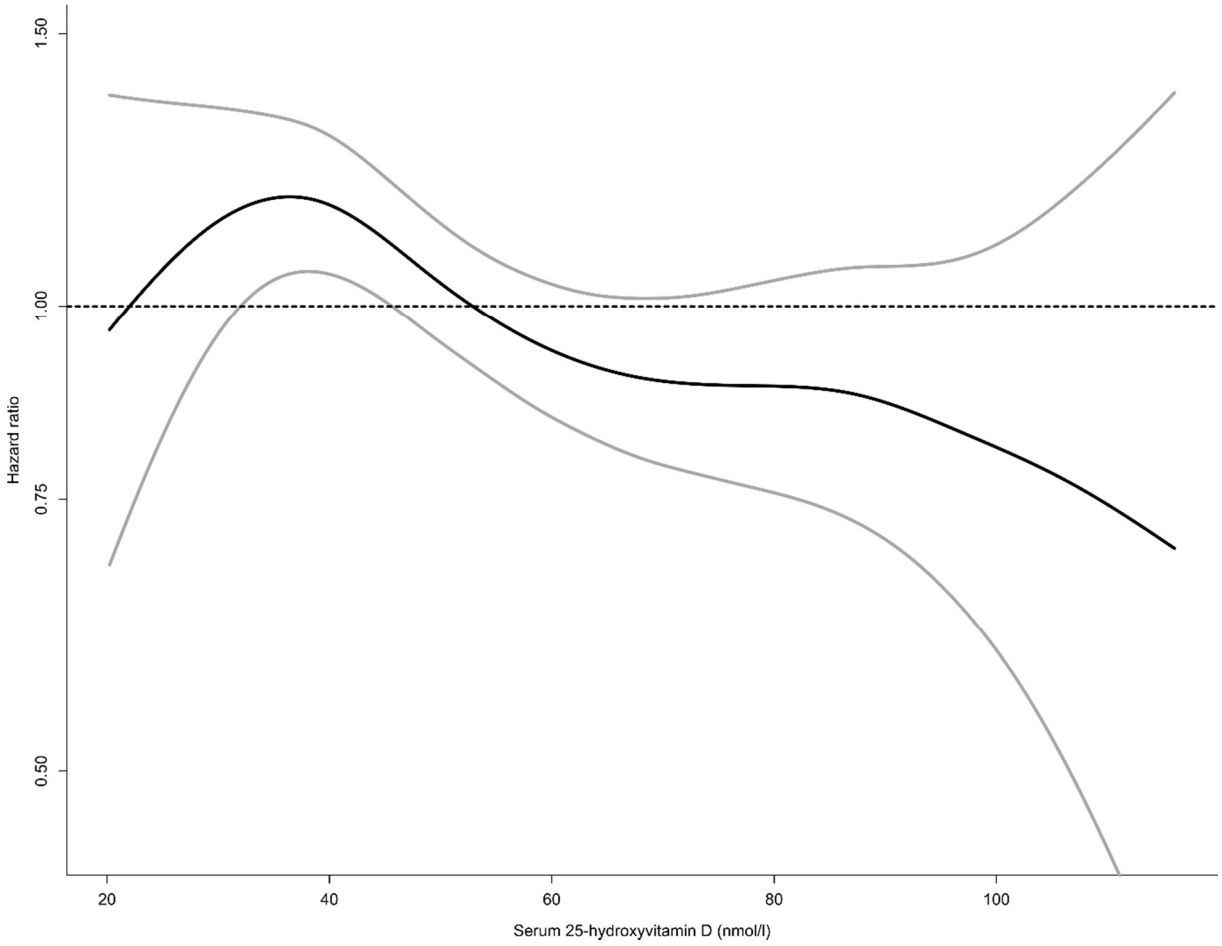
a) Quartiles based on distribution in the subcohorts. Data set restricted to those with valid BMI measurement (1162 cases, 1338 noncases in subcohort)
1) age, gender, and study center
2) age, gender, study center, and BMI
3) age, gender, study center, BMI, month of blood sample

FIG. 1. Inclusion of participants to the case and subcohort groups. A NOREPOS study[1]



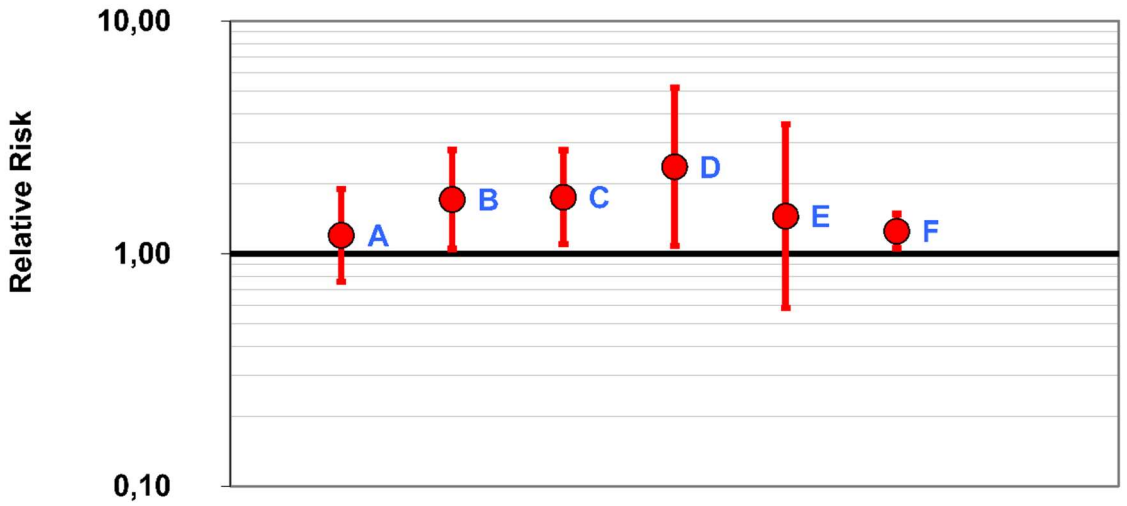
[1] The cases present in the subcohort (n=93 originally sampled; n=84 included in statistical analysis) are duplicates of hip fracture patients occurring in the case group

FIG. 2. Distribution of hazard ratio (solid line) with 95% CI (dotted lines) for hip fracture across the distribution of serum 25-hydroxyvitamin D (nmol/l). A NOREPOS study[1]



[1] Based on Cox proportional hazards regression with penalized splines of s-25(OH)D in a model including age, gender, study center, and BMI, with robust variance estimates and inverse probability weighting for sampling fraction to the subcohort. HR=1 represents average hazard in the data. S-25(OH)D range from 1- to 99-percentile in the subcohort is included.

FIG. 3. Relative risk estimates with 95% confidence intervals for hip fracture at plasma 25-hydroxyvitamin D < 50 nmol/l in prospective studies [1]



[1] Diagram made using Rothman's Episheet (40). A: Study of Osteoporotic Fractures, women, 133 hip fractures, RIA, <47.5 nmol/l vs. >=47.5 nmol/l (33); B: Women´s Health Initiative, women, 400 hip fractures, RIA, <= 47.5 nmol/l (25-percentile) vs. >=70.7 nmol/l (75-percentile) (17); C: NHANES III, men and women, 156 hip fractures, RIA, (15); D: mrOS, men, 81 hip fractures, LC-MS/MS, <= 47.5 nmol/l (25-percentile) vs. >=70.7 nmol/l (75-percentile) (16); E: Uppsala Longitudinal Study of Adult Men, men, 73 hip fractures, HPLC-APCI-MS, 25(OH)D <40 nmol/l (5-percentile) vs. >= 40 nmol/l (14); F: NOREPOS, men and women, HPLC-APCI-MS, 1162 hip fractures.

References

1. **Cheng SY, Levy AR, Lefaivre KA, Guy P, Kuramoto L, Sobolev B** 2011 Geographic trends in incidence of hip fractures: a comprehensive literature review. Osteoporos Int 22:2575-2586

2. **Maggi S, Kelsey JL, Litvak J, Heyse SP** 1991 Incidence of hip fractures in the elderly: a cross-national analysis. Osteoporos Int 1:232-241

3. **Lips P** 2001 Vitamin D deficiency and secondary hyperparathyroidism in the elderly: consequences for bone loss and fractures and therapeutic implications. Endocr Rev 22:477-501

4. **Malavolta N, Pratelli L, Frigato M, Mulè R, Mascia ML, Gnudi S** 2005 The relationship of vitamin D status to bone mineral density in an Italian population of postmenopausal women. Osteoporos Int 16:1691-1697

5. **Saquib N, von Mühlen D, Garland CF, Barrett-Connor E** 2006 Serum 25-hydroxyvitamin D, parathyroid hormone, and bone mineral density in men: the Rancho Bernardo study. Osteoporos Int 17:1734-1741

6. **von Mühlen DG, Greendale GA, Garland CF, Wan L, Barrett-Connor E** 2005 Vitamin D, parathyroid hormone levels and bone mineral density in community-dwelling older women: the Rancho Bernardo Study. Osteoporos Int 16:1721-1726

7. **Garnero P, Munoz F, Sornay-Rendu E, Delmas PD** 2007 Associations of vitamin D status with bone mineral density, bone turnover, bone loss and fracture risk in healthy postmenopausal women. The OFELY study. Bone 40:716-722

8. **Sigurdsson G, Franzson L, Steingrimsdottir L, Sigvaldason H** 2000. The association between parathyroid hormone, vitamin D and bone mineral density in 70-year-old Icelandic women. Osteoporos Int 11:1031-1035

9. **Murad MH, Elamin KB, Abu Elnour NO, Elamin MB, Alkatib AA, Fatourechi MM, Almandoz JP, Mullan RJ, Lane MA, Liu H, Erwin PJ, Hensrud DD, Montori VM** 2011 Clinical review: The effect of vitamin D on falls: a systematic review and meta-analysis. J Clin Endocrinol Metab 96:2997-3006

10. **Cauley JA, Danielson ME, Boudreau R, Barbour KE, Horwitz MJ, Bauer DC, Ensrud KE, Manson JE, Wactawski-Wende J, Shikany JM, Jackson RD** 2011 Serum 25-hydroxyvitamin D and clinical fracture risk in a multiethnic cohort of women: the Women's Health Initiative (WHI). J Bone Miner Res 26:2378-2388

11. **Gerdhem P, Ringsberg KA, Obrant KJ, Åkesson K** 2005 Association between 25-hydroxy vitamin D levels, physical activity, muscle strength and fractures in the prospective population-based OPRA Study of Elderly Women. Osteoporos Int 16:1425-1431

12. **Roddam AW, Neale R, Appleby P, Allen NE, Tipper S, Key TJ** 2007 Association between plasma 25-hydroxyvitamin D levels and fracture risk: the EPIC-Oxford study. Am J Epidemiol 166:1327-1336

13. **van Schoor NM, Visser M, Pluijm SMF, Kuchuk N, Smit JH, Lips P** 2008 Vitamin D deficiency as a risk factor for osteoporotic fractures. Bone 42:260-266

14. **Melhus H, Snellman G, Gedeborg R, Byberg L, Berglund L, Mallmin H, Hellman P, Blomhoff R, Hagström E, Ärnlöv J, Michaëlsson K** 2010 Plasma 25-hydroxyvitamin D levels and fracture risk in a community-based cohort of elderly men in Sweden. J Clin Endocrinol Metab 95:2637-2645

15. **Looker AC, Mussolino ME** 2008 Serum 25-hydroxyvitamin D and hip fracture risk in older U.S. white adults. J Bone Miner Res 23:143-150

16. **Cauley JA, Parimi N, Ensrud KE, Bauer DC, Cawthon PM, Cummings SR, Hoffman AR, Shikany JM, Barrett-Connor E, Orwoll E** 2010 Serum 25-hydroxyvitamin D and the risk of hip and nonspine fractures in older men. J Bone Miner Res 25:545-553

17. **Cauley JA, Lacroix AZ, Wu L, Horwitz M, Danielson ME, Bauer DC, Lee JS, Jackson RD, Robbins JA, Wu C, Stanczyk FZ, LeBoff MS, Wactawski-Wende J, Sarto G, Ockene J, Cummings SR** 2008 Serum 25-hydroxyvitamin D concentrations and risk for hip fractures. Ann Intern Med 149:242-250

18. **Avenell A, Gillespie WJ, Gillespie LD, O'Connell D** 2009 Vitamin D and vitamin D analogues for preventing fractures associated with involutional and post-menopausal osteoporosis (Review). The Cochrane Library: Wiley; 1-120

19. **Abrahamsen B, Masud T, Avenell A, Anderson F, Meyer HE, Cooper C, Smith H, LaCroix AZ, Torgerson D, Johansen A, Jackson R, Rejnmark L, Wactawski-Wende J, Brixen K, Mosekilde L, Robbins JA, Francis RM** 2010 Patient level pooled analysis of 68 500 patients from seven major vitamin D fracture trials in US and Europe. The DIPART (vitamin D Individual Patient Analysis of Randomized Trials) Group. BMJ 340:b5463

20. **Lai JK, Lucas RM, Clements MS, Roddam AW, Banks E** 2010 Hip fracture risk in relation to vitamin D supplementation and serum 25-hydroxyvitamin D levels: a systematic review and meta-analysis of randomised controlled trials and observational studies. BMC Public Health 10:331

21. **Bischoff-Ferrari HA, Willett WC, Orav EJ, Lips P, Meunier PJ, Lyons RA, Flicker L, Wark J, Jackson RD, Cauley JA, Meyer HE, Pfeifer M, Sanders KM, Stahelin HB, Theiler R, Dawson-Hughes B** 2012 A pooled analysis of vitamin D dose requirements for fracture prevention. The New England Journal of Medicine 367:40-49

22. **Borgan Ø, Langholz B, Samuelsen SO, Goldstein L, Pogoda J** 2000 Exposure stratified case-cohort designs. Lifetime Data Anal 6:39-58

23. **Cologne J, Preston DL, Imai K, Misumi M, Yoshida K, Hayashi T, Nakachi K** 2012 Conventional case-cohort design and analysis for studies of interaction. Int J Epidemiol 41:1174-1186

24. **Næss Ø, Søgaard AJ, Arnesen E, Beckstrøm AC, Bjertness E, Engeland A, Hjort PF, Holmen J, Magnus P, Njølstad I, Tell GS, Vatten L, Vollset SE, Aamodt G** 2008 Cohort profile: cohort of Norway (CONOR). Int J Epidemiol 37:481-485

25. **Ahmed LA, Emaus N, Berntsen GK, Bjørnerem Å, Fønnebø V, Jørgensen L, Schirmer H, Størmer J, Joakimsen RM** 2010 Bone loss and the risk of non-vertebral fractures in women and men: the Tromsø study. Osteoporos Int 21:1503-1511

26. **Grønskag AB, Forsmo S, Romundstad P, Langhammer A, Schei B** 2010 Incidence and seasonal variation in hip fracture incidence among elderly women in Norway. The HUNT Study. Bone 46:1294-1298

27. **Gjesdal CG, Vollset SE, Ueland PM, Refsum H, Meyer HE, Tell GS** 2007 Plasma homocysteine, folate, and vitamin B 12 and the risk of hip fracture: the hordaland homocysteine study. J Bone Miner Res 22:747-756

28. **Tsugawa N, Suhara Y, Kamao M, Okano T** 2005. Determination of 25-hydroxyvitamin D in human plasma using high-performance liquid chromatography--tandem mass spectrometry. Anal Chem 77:3001-3007

29. **R core team** 2013 The R Project for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria. http://www.R-project.org

30. **Breslow N** 2012 "cch: Fits proportional hazards regression model to case-cohort data". Function in Therneau T: "Package 'survival': Survival analysis, including penalised likelihood". http://cran.r-project.org/web/packages/survival/survival.pdf

31. **Therneau T, Grambsch PM** 2000 Modeling Survival Data. Extending the Cox Model. 1st ed. Springer Science

32. **Wang Y, Jacobs EJ, McCullough ML, Rodriguez C, Thun MJ, Calle EE, Flanders WD** 2009 Comparing methods for accounting for seasonal variability in a biomarker when only a single sample is available: insights from simulations based on serum 25-hydroxyvitamin D. Am J Epidemiol 170:88-94

33. **Cummings SR, Browner WS, Bauer D, Stone K, Ensrud K, Jamal S, Ettinger B** 1998 Endogenous hormones and the risk of hip and vertebral fractures among older women. Study of Osteoporotic Fractures Research Group. N Engl J Med 339:733-738

34. **Snellman G, Melhus H, Gedeborg R, Byberg L, Berglund L, Wernroth L, Michaëlsson K** 2010 Determining vitamin D status: a comparison between commercially available assays. PLoS One 5:e11555

35. **Farrell CJ, Martin S, McWhinney B, Straub I, Williams P, Herrmann M** 2012 State-of-the-art vitamin D assays: a comparison of automated immunoassays with liquid chromatography-tandem mass spectrometry methods. Clin Chem 58:531-542

36. **Ensrud KE, Ewing SK, Fredman L, Hochberg MC, Cauley JA, Hillier TA, Cummings SR, Yaffe K, Cawthon PM** 2010 Circulating 25-hydroxyvitamin D levels and frailty status in older women. J Clin Endocrinol Metab 95:5266-5273

37. **Lips P** 2007 Vitamin D status and nutrition in Europe and Asia. J Steroid Biochem Mol Biol 103:620-625

38. **Jorde R, Sneve M, Hutchinson M, Emaus N, Figenschau Y, Grimnes G** 2010 Tracking of serum 25-hydroxyvitamin D levels during 14 years in a population-based study and during 12 months in an intervention study. Am J Epidemiol 171:903-908

39. **Carter GD** 2012 25-hydroxyvitamin D: a difficult analyte. Clin Chem 58:486-488

40. **Rothman KJ, Greenland S, Lash TL** 2008 Modern Epidemiology. 3rd ed. Philadelphia: Lippincott Williams and Wilkins